IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 8511
Rebekka M. Wachter et al.	Examiner: Nashaat T. Nashed
Application No.: 10/620,099	Technology Center/Art Unit: 1656
Filed: July 14, 2003	DECLARATION UNDER 37 C.F.R. 1.132
For: LONG WAVELENGTH ENGINEERED FLUORESCENT PROTEINS	
Customer No.: 20350	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, George Hanson, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. §1001, and that they may jeopardize the validity of the referenced patent application or any patent issuing thereon, declare and state as follows:

1. All statements herein made of my own knowledge are true and statements made on information or belief are believed to be true.

2. I am currently a scientist with Invitrogen, Inc., a licensee of the application, and enclose a copy of my resume (*see*, Exhibit A) which sets forth my credentials as an expert.

3. I have read U.S. Patent Application Serial No. 10/620,099 (hereinafter, the '099 application) of which Dr. Rebekka M. Wachter is the first named inventor.

4. I have read the Office Action mailed from the U.S. Patent Office on October 22, 2007 in the '099 application.

5. I understand that the invention disclosed and presently claimed in the '099 Application (herein "the invention") is, at its broadest, an engineered fluorescent protein whose

amino acid sequence is at least 85% identical to the amino acid sequence of *Aequorea* green fluorescent protein (GFP) and which differs at least by comprising the substitutions T203Y, S65G, S72A, V68L, and H148(R, G, Q, A, N, or K) and by at least one substitution selected from the group consisting of R96K; Q183N or K; V150S, T, Q, N A, C, M, G or L; I152L,V, F, S, T, Q, N A, C, M, or G; F165Y, W, or L; H181F, W, K or R; Q183R, N, S, or C; L201S, T, Q, N, V, I, A, C, M, or G.

6. I understand that claims 143-146, 148-153, 188, 189, 191, and 193 of the '099 Application stood rejected under 35 U.S.C.112, first paragraph, as allegedly embracing subject matter which was not enabled at the time the application was filed.

7. With respect to the above grounds for rejection, I next address the rejection based upon an alleged lack of enablement.

8. A statement incorporating by reference all the publications and patent documents referenced in the specification is found in the last three lines of text on page 86.

9. The claimed invention speaks to an *Aequorea*-related fluorescent protein (herein "*Av*GFP-rp") "whose amino acid sequence is at least 85% identical to the amino acid sequence of *Aequorea* green fluorescent protein" as set forth in pending base claims 143 and 144. An *Av*GFP-rp of the claims would therefore require that the polypeptides have fewer than 36 amino acid differences, with respect to the wild type GFP protein. I provide evidence here that the '099 application does enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to practice the invention commensurate in scope with these claims. Specifically, I establish how the '099 specification supports the construction of an *Av*GFP-rp having 85% sequence identity relative to wt *Aequorea* GFP, and which would retain useful, if not necessarily improved, fluorescent activity.

The Office Action is concerned about the amount of experimentation to practice a large number of mutations (i.e., 85% sequence identity) in any one Av-GFP-rp. Direct evidence for the advanced state of the art in this field can be found in the teachings of Campbell et al. (*see*, Exhibit I : Campbell et al., PNAS, 99:7877-7882 (2002)). This reference demonstrates the ability to engineer 33 mutations into a GFP-related protein isolated from *Discosoma* coral, dsRed. The mutant generated, which has 85% sequence homology to the wild type dsRed protein, not only retains useful fluorescent activity, but demonstrates advantageous shifts in its emission and excitation peaks, with respect to the wild type protein.

Even more powerful evidence for the ability of persons of ordinary skill in the art to re-engineer the GFP comes from Lawrence et al. (J. Am. Chem. Soc. 129:10110-10112 (2007), *see*, Exhibit J). Starting from a modified GFP which has about 7 substitutions already from the wild-type GFP, *they re-engineered several GFP's having up to an additional 36 amino acids non-conservatively substituted (i.e., negatively charged or neutral amino acids were replaced by an Arg or Lys)*. In another of the modified GFP proteins, 15 negatively charged or neutral amino acids were replaced by a Glu or Asp. The Lawrence et al. reference clearly exemplifies that the amount of effort required to practice claims of the present scope is not undue, but well within current practice in the art. Accordingly, by almost any criteria, the state of the art in this field is impressively high.

The teachings of the specification further support their enablement. To start, the specification teaches *Av*GFP-rps containing a mutation at T203, which may further comprise mutations at S65 and Y66 (*see*, specification, the full paragraph on p. 6), as well as H148 (*see*, first full paragraph of specification on p. 13). The specification also discloses the high-resolution crystal structure of wt GFP (see figure 1) and also the coordinates for the structure of the same protein containing a single point mutation at residue S65 (*see*, figure 5). As stated in the first full paragraph at p. 29 of the specification, "(t)his information is useful in identifying amino acids whose substitution alters fluorescent properties of the protein." Accordingly, the specification next sets forth several classes of residues which can be mutated in order to alter such properties. Furthermore, the specification proceeds to teach the function of each residue and the specific mutations that could be made for each. These teachings, in combination with the high-resolution crystal structure of GFP, is sufficient to enable one of ordinary skill in the art to engineer combinations of the following mutations in an *Av*GFP-rp without undue experimentation and with a very high expectation of success.

In the second paragraph at p. 29, lines 9 to 11, the specification teaches that "amino acids that are within about 0.5 nm of the chromophore influence the electronic environment of the chromophore." And at p. 30, starting at line 9, the specification teaches that "Table B lists several amino acids located within about 0.5 nm from the chromophore whose substitution can result in altered fluorescent characteristics. The table indicates, underlined, preferred amino acid substitutions at the indicated location to alter a fluorescent characteristic of the protein." Residues indicated in Table B on page 30 inelude L42, V61, T62, V68, N121,

Y145, H148, V150, F165, I167, and T203. Further particular such residues that can be substituted with polarizable side groups are found in Table C on page 32 and include Q69, Q94, Q183, and N185.

In the paragraph starting at line 20 on p. 32, the specification also teaches that "an amino acid that is close to a second amino acid within about 0.5 nm of the chromophore can, upon substitution, alter the electronic properties of the second amino acid, in turn altering the electronic environment of the chromophore." Particular residues are set forth in Table D on page 32 and include L220 and V224.

Additionally, the specification teaches that "anion binding can be improved by creating more and or tighter binding interactions between the anion of interest and polar groups within the binding pocket." (see paragraph starting on line 14 at p.35) Particular residues are listed in Table E on page 35 and include Q69, R96, and Q183. Moreover, the specification recites that "(t)he development of higher affinity anion binding sites therefore requires the creation of sufficient ion-protein interactions for example by the substitution of hydrophobic residues that line the ion binding pocket with more polar residues with more hydrogen bonding potential. Positions for these type of substitutions for improving the ion binding for larger and smaller anions are presented in Table F." (*see,* paragraph just above the table on page 36) Residues listed in Table F on page 36 include V150, I152, V163, F165, H181, Q183, and L201.

The specification teaches that *Av*GFP-rps include a number of engineered proteins included in Table A (*see,* page 26). This table contains a number of mutational combinations that were engineered for specific utilities, be they shifted excitation and or emission spectra, increased quantum yields, greater relative fluorescence, or otherwise. Clone W2, found in said table, consists of an *Av*GFP-rp with seven specific mutations; Y66W, I123V, Y145H, H148R, M153T, V163A, and N212K.

Further, the specification teaches that *Av*GFP-rps include "variants that include one or more folding mutations" (*see,* first paragraph on p. 28) as defined in the next paragraph as F64L, V68L, S72A, T44A, F99S, Y145F, N146I, M153T or A, V163A, I167T, S205T, and N212K. These mutations "improve the ability of fluorescent proteins to fold at higher temperatures, and to be more fluorescent when exposed in mammalian cells, but have little or no effect on the peak wavelengths of excitation and emission." (*see,* second paragraph on p. 28).

The specification also teaches that *Av*GFP-rps include "allelic variants of this sequence, e.g., Q80R... (M. Chalfie et al., Science, (1994) 263:802-805)" (*see,* paragraph bridging pp. 25 and 26). Furthermore, additional cited art which was thereby incorporated by reference (*see,* paragraph 2 at p. 25) discloses several other allelic variants that display biophysically equivalent function with respect to the peak wavelengths of excitation and emission. Prasher et al., cited at p.1, line 22 and thereby incorporated by reference, discloses the isolation of several isoforms of wild type *Aequorea victoria* GFP from varying cDNA libraries (*see,* Exhibit B : Prasher et al., Gene 111:229-233 (1992)). They report a comparative analysis of two of these clones, *gfp2* and *gfp10* which demonstrates that sequence differences in the GFP protein exist in the natural population. Notably, four conservative differences exist between *gfp10*, which is the sequence used in the instant application, and *gfp2*, isolated from a cDNA library generated from jellyfish collected at Friday Harbor in Washington. These conservative mutations consist of F100Y, T108S, L141M, and V219I (*see,* Table I of Prasher et al.).

Furthermore, art (Tsien et al., Ann. Rev. Biochem. 67:509-544 (1998), *see,* Exhibit C) which was cited at page 85, line 8 of the instant application and thereby incorporated by reference, identifies other sites in which complementary mutations can be generated to promote binding interactions between two GFPs. In this reference, Tsien et al. teach that a number of amino acids contribute to the dimmer interface of the protein. These residues include A206, L221, F223, Y39, E142, N144, S147, N149, Y151, R168, N170, E172, Y200, S202, Q204, and S208. Persons of ordinary skill would recognize that such locations would not likely be critical to the operability of the protein as a fluorescent protein and would be locations where conservative substitutions would be especially well tolerated with respect to retaining fluorescent activity. The specification of the '099 application teaches that "(c)onservative amino acid substitutions providing functionally similar amino acids are well known in the art." (*see,* p. 21). Groups of amino acids that are conservative substitutions for one another can be found in paragraphs 93 to 98 of the instant application.

Finally, Dopf and Horiagon (*see,* Exhibit D : Dopf and Horiagon, *Gene*, 173:39-44 (1996)), which is incorporated by virtue of its reference at lines 1-3 of p. 59, teach that residues 1 and 233-238 are not required for the characteristic emission and absorption spectra of native GFP (*see,* Dopf and Horiagon summary on page 39), evidencing that mutation of these residues would not effect the fluorescent activity of an *Av*GFP-rp. This finding is corroborated

by the crystal structure of GFP (*see,* figures 1 and 5 of the specification), which shows no electron density for these residues, evidencing that they are unordered and therefore unlikely to contribute to the chromophore's electron environment.

Taking these teachings all together, the specification therefore provides support for up to 61 individual mutations (residues 1, 39, 42, 44, 61, 62, 64, 65, 66, 68, 69, 72, 80, 94, 96, 99, 100, 108, 121, 123, 141, 142, 144, 145,146, 147, 148, 149, 150, 151, 152, 153, 163, 165, 167, 168, 170, 172, 181, 183, 185, 200, 201, 202, 203, 204, 205, 206, 208, 212, 219, 220, 221, 223, 224, 233, 234, 235, 236, 237, and 238) concerning more than 25% of the wildtype GFP sequence.

Further to this analysis, in the '099 application teaches how to easily generate and select mutations that provide a desired result. First, Heim et al., 1994 (incorporated by reference by virtue of its citation at page 2, lines 18 and 19; *see,* Exhibit E : Heim et al., PNAS-USA 91:12501-12504 (1994)), teach that mutants can be generated by standard means, namely hydroxylamine treatment or error prone PCR, and screened for different emission colors through the use of a xenon lamp and grafting monochromator. In this fashion five mutant clones were identified as containing at least one amino acid change that consequences in the desired result.

Secondly, Heim et al., 1995 (incorporated by reference via its citation at lines 1 to 3 on p. 3; *see,* Exhibit F : Nature 373:663-664 (1995)), demonstrate that hypothesis-driven site-specific mutation of a single residue can also generate a desired result. In this study, Heim et al. hypothesized that Ser 65, which becomes part of the p-hydroxybenzylidencimidazolinone chromophore, would undergo a dehydration event resulting in a vinyl side chain, which would contribute to the overall properties of said chromophore. Therefore, mutation of this residue, which would result in exclusion of the vinyl formation, should result in formation of a chromophore with altered spectral properties. This is indeed what was found when Ser 65 was mutated. Furthermore, the resulted mutant *Av*GFP-rp displayed advantageous spectral properties. It should be noted that this hypothesis was made prior to the determination of the high-resolution crystal structure of the protein, and therefore future site specific mutations should be considerably easier to design due to the availability of many such structures.

Delagrave et al., incorporated by reference by virtue of its citation at page 62, line 24, teach additional methods for generating and screening such mutant *Av*GFP-rp's (*see,* Exhibit G : Delagrave et al. Biotechnology, 13:151-155 (1995)). This reference teaches the use of

optimized combinatorial mutagenesis techniques and Digital Imaging Spectroscopy to isolate mutant GFP proteins that show red-shifted excitation spectra. Using these methods, Delagrave et al. were able to screen by fluorescence "Thousands of colonies on Petri dishes" (*see*, the first complete paragraph on page 152) with "colony densities ranging from 100 to 900 colonies/plate" (*see*, Digital Imaging Spectroscopy section on page 154 thereof). The result of the screen was the isolation of seven mutant proteins with the desired red-shifted excitation spectra, that each contained between three and four changes in the amino acid sequence.

Finally, Ehrig et al., also incorporated by reference virtue of its citation at page 61, line 31, teaches a fifth method for generating mutant *Av*GFP-rp's with a desired property (*see*, Exhibit H : Ehrig et al., FEBS Letters 367:163-166 (1995)). In this paper, Ehrig et al. were able to generate mutant proteins that differentially excited at one, but not the other, of the two characteristic maximal excitation wavelengths of wild type GFP. In order to do so, they randomly mutagenized GFP cDNA through use of the *E. coli* XL1-Red strain and subsequently screened approximately 200,000 colonies for the desired separation of excitation wavelengths. The result of the study was the isolation of *Av*GFP-rp's with point mutations in one of two residues, which conferred to the respective protein the ability to excite at either 390 or 470 nm, but not both.

The above four references, each incorporated by reference into the specification, teach five different methods for generating additional point mutants of *Av*GFP-rp's. These references demonstrate that any person skilled in the art to which it pertains, or with which it is most nearly connected, should easily be able to generate additional mutations in the mutant *Av*GFP-rp's taught in the '099 specification. Although the '099 specification alone is sufficient for any person skilled in the art to which it pertains to practice the full scope of claimed invention, these four teachings were able to generate their respective mutations without the aid of a high-resolution crystal structure as set forth in Figures 1 and 5 of the specification. The Applicants teaching of this structural details greatly simplifies the task of obtaining additional beneficial and/or silent mutations in *Av*GFP-rps.

In addition, I also state that one of ordinary skill in the art would easily be able to design a multitude of conservative mutations, especially in residues distal to the chromophore, those whose side chains are solvent exposed and do not contribute directly to the electron environment of the chromophore, and those found in loop regions connecting the beta-strands in

the ternary structure. This expectation is in accord with the conservative mutations identified by Prasher et al. and Dopf et al., as discussed above. I also note that Heim et al., which was discussed above and is incorporated by reference as mentioned, in their discussion of GFP mutants with altered spectral properties, disclosed that they sequenced and recombined the sequences of these mutants to eliminate the neutral mutations which went unreported (*see*, Exhibit E : Heim et al., PNAS-USA 91:12501-12504 (1994) at p.12502 right column, starting at the ninth line of text).

Additional evidence for these types of silent mutations can be found in U.S. Patent No. 5,625,048, which discloses seven mutations; K3R, D76G, F99I, N105S, E115V, T225S, and K238E which had no significant impact on the properties of their corresponding *Aequorea* green fluorescent proteins (*see,* Exhibit K, U.S. Patent No. 5,625,048, which matured from U.S. Patent Application Serial No. 08/337,915, at col. 5, second full paragraph, lines 47 to 54) Furthermore, the dearth of literature reporting on silent mutations can not be fairly construed to indicate that such do not exist or are not readily available. Rather, it is generally the case that generated conservative and or silent mutations are not likely to be reported for a number of reasons. Most importantly, such changes generated during mutational and screening analysis are not sought for and rarely sequenced because they do not confer altered properties to the resulting mutant protein and because they add comparatively little to the understanding or the practical application of the fluorescent proteins.

Given the guidance provided above and in the '099 specification, the current abundance of structural information available, the demonstration of large-scale mutagenesis, the availability of conservative and/or silent mutations, and the demonstrated ability of persons in the art of a GFP protein engineering to construct similar proteins with the recited amount of sequence identity, the experimentation needed to practice the full scope of the claimed invention is well within an amount which would be routine in its art.

10. The declarant has nothing further to add.

George Hanson — Date: April 4, 2008

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